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Our research has seen the completion of several projects aimed at elucidating the chronopharmacological properties of benzodiazepines in rodents and monkeys, and at investigating the circadian and homeostatic mechanisms involved in the regulation of sleep in monkeys. We have also recently completed a series of studies on the neural control of circadian rhythms in the squirrel monkey. The results obtained thus far have several interesting implications, some of which are currently being tested.

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Phase Response Curve (PRC)
Phase Shift
Reentrainment
Sleep Deprivation (SD)
Homeostasis.

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M.C. Moore-Ede

FINAL TECHNICAL REPORT

AIR FORCE OFFICE OF SCIENTIFIC RESEARCH

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1. CHRONOPHARMACOLOGICAL PROPERTIES OF BENZODIAZEPINES

a. Phase-Shifting Effects of Diazepam on Circadian Activity Rhythms in Hamsters

Several recent studies indicate that benzodiazepine administration can reset circadian rhythms in rodents, but the mechanisms involved are not completely understood as yet. Specifically, it is important to determine whether these drugs are acting on the central pacemaker directly (i.e., the suprachiasmatic nuclei of the hypothalamus, or SCN) or on input pathways that relay light information to the pacemaker, particularly the retinohypothalamic tract (or RHT). An additional possibility is that these drugs are directly affecting behavioral activity which in turn is resetting the central pacemaker through some as yet unspecified feedback pathway. Evidence for such feedback effects was reported by Turek and colleagues at the recent meeting of the Society for Research on Biological Rhythms (1988). They found that the phase-advancing effects of triazolam in hamsters were blocked when the hyperactivity usually induced by triazolam injections was prevented by restraining the animals.

Our initial studies focused on the phase-resetting effects of the long-acting benzodiazepine, diazepam. We first obtained a complete phase response curve (PRC) for 12.5 mg/kg injections of diazepam in hamsters maintained in constant illumination (LL). The PRC shows that diazepam causes phase advances of the hamsters' circadian rhythm of locomotor activity (computer-monitored wheelrunning) when administered during the animals' subjective day (i.e., during their inactive phase) and phase delays when administered during subjective night. The PRC was very similar to the PRC for dark pulses, suggesting that diazepam may be acting by blocking the transmission of light input to the circadian pacemaker.

In order to test this hypothesis, we obtained a second PRC for the same dose of diazepam in optically-enucleated hamsters. The results showed that blinding almost completely blocked the phase-resetting effects of diazepam, indicating that this drug may indeed be acting on light input pathways rather than on the pacemaker itself. We also obtained a

dose-response curve for diazepam in enucleated hamsters, with doses ranging from .5 to 100 mg/kg. The injections were administered during the delay portion of the PRC, but none of the doses tested caused any significant phase shifts, indicating that this effect was not dose-dependent. It should be noted that diazepam, like triazolam, often causes hyperactivity in both sighted and blinded hamsters. However, the fact that blinded hamsters did not show any phase shifts is evidence that the resetting effects of diazepam are not attributable to a feedback effect of increased behavioral activity.

b. Phase-Shifting Effects of Triazolam on Circadian Activity Rhythms in Squirrel Monkeys

We recently completed a phase response curve for the short-acting benzodiazepine, triazolam, in squirrel monkeys maintained in LL. A dose of .2 mg administered during mid- to late subjective day (Circadian Time 6-10 or CT 6-10) caused phase advances, while the same dose administered during mid- to late subjective night (CT 17-24) caused phase delays. Lasting changes in the period of the free-running rhythms also followed about half of the triazolam injections, although these did not appear to be related to the phase of injection or to the size and direction of the phase shifts. Injections of the DMSO vehicle alone failed to produce phase shifts, although period changes did occur on three occasions. A dose-response profile at CT 6-10 is currently being completed for triazolam doses of .05 mg, .1 mg, .15 mg and .2 mg. Results to date show a positive relationship between dose and size of phase shift (all phase advances). Unlike the case in hamsters, triazolam causes sedation in squirrel monkeys rather than hyperactivity. This observation clearly demonstrates that the phase-resetting action of triazolam is not an indirect effect of increased behavioral activity.

These results provide the basis for specific predictions about the optimal timing of triazolam administration for accelerating the reentrainment of circadian rhythms following phase shifts of the daily LD cycle. In the case of advancing phase shifts, we expect that a single injection of triazolam around CT 7 (i.e., 7 hrs after light onset) on the day of the phase shift will lead to a faster rate of reentrainment than no injection or than injections at any other time, while in the case of delaying phase shifts, maximal reentrainment rates should follow triazolam administration at CT 18-24. We plan to test these predictions in squirrel monkeys in the coming months.

2. BENZODIAZEPINE RECEPTOR BINDING STUDIES

Receptor binding studies can provide valuable information about the mechanisms of action of benzodiazepines in the nervous system, as they provide a biochemical basis for the observed behavioral effects. In

biochemical studies, brain regions are dissected from animals at intervals across the day. The tissue is homogenized and incubated with tritiated benzodiazepines to characterize diurnal fluctuations in receptor density and/or affinity. Earlier studies from the laboratory showed that, in the rat, benzodiazepine receptor binding exhibits daily rhythmicity in the frontal lobe and in the cerebellum, but not in the hypothalamus or the medulla/pons region. The rhythms were entirely due to rhythmic changes in receptor density rather than in affinity. A daily rhythm in GABA receptor density was also demonstrated in the frontal lobes. The rhythms were eliminated after lesions of the SCN. These data suggest that GABA/benzodiazepine receptors may be involved in the transmission of temporal information from the SCN to other parts of the brain.

We recently repeated the benzodiazepine receptor binding study in hamsters. With six animals at each of six timepoints, we found no significant benzodiazepine receptor rhythm in cortex, cerebellum, striatum or in light- or dark-adapted retinae. We are currently repeating this study in rats in order to determine whether the absence of daily rhythms in hamsters reflects species or procedural differences.

The biochemical studies just described provide information about gross changes in receptor density in large and probably heterogeneous brain regions. A more detailed picture, however, can be obtained with histochemical procedures in which thin brain sections are incubated in tritiated benzodiazepines and other compounds of interest, and exposed to film for several weeks. The resulting photographic images are digitized onto a computer, where image analysis and reconstruction can pinpoint the anatomical distribution of drug and neurotransmitter receptors. We are currently using this procedure to study receptors in the SCN in both hamsters and squirrel monkeys. Initial results indicate a low density of benzodiazepine receptors in the hamster SCN relative to surrounding hypothalamus, with no significant day-night variation.

3. CIRCADIAN AND HOMEOSTATIC REGULATION OF PRIMATE SLEEP-WAKE CYCLE

The regulation of sleep in a wide range of species, including humans, involves circadian as well as homeostatic recovery processes. We recently completed a series of studies aimed at evaluating the relative contributions of these two factors in the squirrel monkey, in preparation for studying the mechanisms of resetting of the primate sleep-wake cycle by benzodiazepines and other pharmacological agents. This was done by studying multiple sleep characteristics, including timing, duration, composition and cortical EEG power content in different frequency ranges, in undisturbed animals in LD and LL, and in animals subjected to sleep deprivations in LL for specific durations and terminating at specific circadian phases. Six sleep deprivation durations were studied: 0^{o}, 90^{o},

180°, 360°, 450° and 540°, where 360° corresponds to the period of an animal's free-running rhythm (about 25h). All sleep deprivations (SDs) started one hour before the predicted time of consolidated sleep (CS) onset. Thus, in the 0° and 360° conditions, animals were allowed to sleep at the same circadian phase so that any differences in sleep characteristics would be attributable to purely homeostatic processes. The same is true of the 90° and 450° conditions, and the 180° and 360° conditions. Comparisons between these 3 pairs of conditions would then reveal any circadian influences on sleep.

The timing of sleep after SD was strongly dependent on circadian phase. Sleep latency was shortest after SDs ending halfway through the predicted consolidated sleep episode (90° and 450°), slightly longer after SDs ending at the predicted time of consolidated sleep onset (0° and 360°), and longest after SDs ending at the predicted time of consolidated wake onset (180° and 540°), with most animals remaining awake until the next predicted time of consolidated sleep, despite the fact that animals in the 540° condition had been without sleep for two whole days.

Consolidated sleep duration was also highly dependent on circadian phase. The duration of the first recovery sleep episode was of approximately normal length after 0°, 360°, 180° and 540° SDs, but shorter after 90° and 450° SDs. The proportion of consolidated sleep time spent in REM and NREM sleep during recovery from SD was unchanged. The influence of homeostatic recovery processes appeared to be limited to a small increase in daytime napping after SD.

Quantitative analysis of the cortical EEG data recorded from the monkeys before and after SD is not yet complete, but the results obtained thus far have already revealed additional contributions of both circadian and homeostatic processes. Thus, cortical EEG power in the 0.5-2.0 Hz range (i.e., the delta wave range) was found to exhibit circadian rhythmicity under both entrained and free-running conditions. This rhythmicity was accounted for entirely by rhythmic changes in delta wave density and not in delta wave amplitude which remains constant throughout the circadian cycle. During recovery from SD, however, EEG delta power increases as a result of increases in both delta wave density and amplitude.

In summary, our results indicate that the timing and duration of sleep in the squirrel monkey are under strong circadian control which allows only minor compensatory changes following sleep deprivation. Some of the lost sleep, however, is compensated for by an increase in sleep intensity resulting from increases in the density of delta waves as well as in their amplitude. These data point to the necessity of quantitative EEG analyses for a complete picture of the effects of

chronopharmacological agents, particularly agents with hypnogenic properties like the benzodiazepines.

4. CIRCADIAN RHYTHMS OF NEURAL ACTIVITY

One of the most direct approaches for determining the effects of pharmacological agents on circadian processes is to record the neural activity of the circadian pacemaker(s) before and after drug administration. The feasibility of this approach is demonstrated in a series of studies recently completed in the squirrel monkey in which continuous, long-term recordings of neural multiple unit activity (MUA) were obtained from many different brain regions, including the SCN. Most brain regions exhibited clear circadian rhythms in MUA under both entrained and free-running conditions. Different regions showed differences in such circadian characteristics as phase, waveform and amplitude, as well as in the presence and magnitude of ultradian components related to behavioral activity, light exposure and sleep stages.

Circadian rhythms recorded from the SCN consisted of a smooth sinusoidal function with a gradual increase anticipating lights-on and reaching maximum levels during the animals' subjective day. Superimposed on this were sharp peaks in MUA distributed over the light phase of the LD cycle. These peaks disappeared when the monkeys were kept in constant darkness for a day, indicating that they reflected changes in light input reaching the SCN resulting from changes in the animals' behavior (i.e., short daytime naps or periods of quiescence with eyes closed alternating with periods of increased behavioral activity).

Also of interest were rhythms recorded from the preoptic area and the basolateral nucleus of the amygdala--areas that have been implicated in sleep regulation. Unlike other brain regions, these showed circadian rhythms with peak levels during the animals' subjective night, apparently reflecting the presence or absence of NREM sleep.

We are currently adapting our MUA recording system for use in rodents, with the aim of monitoring SCN activity before, during and after systemic administration of benzodiazepines. We also plan to record SCN activity in rats implanted with cannulas terminating in the vicinity of the SCN to determine whether or not local administration of benzodiazepines in the SCN can alter the neural activity of the circadian pacemaker. These procedures will allow us to examine directly the relation between SCN neural activity and any phase-resetting effects of benzodiazepines and other pharmacological agents. In the case of drugs with hypnogenic properties, recordings will also be made from the preoptic and basolateral amygdala areas to determine their effects on sleep-related neural activity.

5. PERSONNEL ASSOCIATED WITH THE RESEARCH EFFORT

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